IMPROVEMENT OF ENDOTHELIAL CELL-CELL COHESION

Related Applications

This application claims priority of U.S. Serial Nos. 60/241,216 and 60/243,693, the contents of both of which are incorporated by reference.

The research leading to the present invention was supported, at least in part, by Grant ID#1 KO8 HLO4166-01 from NIH. Accordingly, the Government may have certain rights in the invention.

Background

This invention relates to the field of coating graft surfaces with cells or inducing native cells to migrate on an implanted device or graft.

In humans, when an artificial biomaterial (prosthetic) bypass graft is placed in the circulation, the vessel's endothelial cells do not spread on the surface to completely heal the graft. Since the endothelium produces a variety of substances that inhibit blood clotting, the absence of an endothelium is problematic. This lack of endothelial healing in man is unique. Cows, pigs, dogs and primates are able to endothelialize grafts by developing a biologic lining consisting of smooth muscle cells, endothelial cells, and matrix protein on the flow lumen of the graft, shielding it from the blood. But this does not occur in man. Attempts to line prosthetic grafts prior to transplantation with human endothelial cells have not been successful because the cells don't attach well and undergo shear-induced detachment.

We have determined that human endothelial cells derived from arteries lack a certain property that renders them more prone to shear stress induced detachment. Moreover, we have identified that the surface expression of the family of proteins responsible for this behavior is substantially reduced as compared with bovine cells. The goal of this invention

is to restore function to human vascular endothelial cells by modulating the presence of this factor on the cell surface. Inducing the cells to attach to each other by improving cell-cell cohesion, rather than focussing on attachment to the graft, is a goal of this invention.

Summary of the Invention

In a general aspect, the invention is a method for populating a solid surface with cells by increasing the cell-to-cell cohesion of said cells. A test for cell-to-cell cohesion is the test for cell cohesivity described in Example 2. This may be done by increasing the amount of cadherin per cell. Another method for so doing comprises reducing the amount of dissociation of cadherin from the cytoskeleton of said cells. As discussed below, this may be done by various means, but in particular by reducing or eliminating the phosphorylation of a molecule associated with the adherens junction between the cells.

All aspects of this invention may be performed with any cell which can be modified to provide increased cohesivity, including cells which may be engineered for this purpose. Examples of particularly useful cells include epithelial cells, endothelial cells, in particular vascular endothelial cells, muscle cells such as smooth muscle cells, fibroblasts, mesenchymal cells, and nerve cells. Other cells which are particularly useful in this invention are cells which naturally or by engineering produce a given compound or exhibit a given behavior. For example a cell which naturally or by engineering produces heparin may be increased in cohesivity by the methods of this invention, to provide a solid surface such as a bypass graft lined with cells which produce heparin to prevent blood clotting. Cells of this invention may be obtained from any source, preferably a eukaryotic source. Human cells are preferred, but nonhuman cells are also contemplated for any use of this invention. Thus, any discussion of cells below, even if applied to a specific cell type such as a human vascular endothelial cell, may be taken to apply to nonhuman cells of the same cell type, and also to human and nonhuman cells of other cell types (with regard to discussion of specific experiments performed with specific cells in the Examples -- such experiments may be performed with other cell types, but the specific results discussed apply to the cells with which the experiments were performed).

One embodiment of the invention is a method of populating a solid surface with human vascular endothelial cells, said process comprising increasing the cell-to-cell cohesion of said endothelial cells.

One aspect of the above method is populating a solid surface with human vascular endothelial cells, by reducing the amount of dissociation of cadherin from the cytoskeleton of said human vascular endothelial cells. "Reducing" means reducing the amount of dissociation to a point measurably below natural levels and includes reducing dissociation to the point of elimination. Reduction of dissociation may be accomplished by any physical, electrical, chemical or pharmacological means known in the art, including genetic engineering means of the molecules involved. In particular dissociation may be reduced by reducing or eliminating the phosphorylation of a molecule associated with the adherens junction between the human vascular endothelial cells. An example of such a molecule is β catenin. Phosphorylation of β catenin reduces cadherin association with the cytoskeleton, and methods of phosphorylating β catenin are known. It is part of this invention that phosphorylation of β catenin increases cell-cell cohesion in human vascular endothelial cells. Phosphorylation may reduced or eliminated by use of any agent known to do so, i.e. any agent which is known to modify phosphorylation, in an amount effective to reduce or eliminate phosphorylation. Examples of such agents include corticosteroids, and enzyme inhibitors such as serine-threonine kinase inhibitors, protein tyrosine kinase inhibitors, and phosphatase inhibitors. Specifically, such agents include 6-dimethylaminopurine, stuarosporine, erbstatin, herbimycin A, genestein, tyrophostins, and vanadate.

In a related aspect, the invention is a method of populating a solid surface with human vascular endothelial cells, said process comprising increasing the amount of cadherin per cell. Any cadherin (eukaryotic, preferably mammalian, more preferably human) may be used in this invention, but preferred cadherins are VE, N, E, and P cadherin. Preferably this is accomplished by increasing the number of expressible cadherin genes in the endothelial cells so as to result in the increased production of functional protein. One means of so doing is putting into the cells a gene expressibly encoding cadherin, for example by transfection, electroporation, and other known means.

In a particular aspect, the invention is a method of increasing cell-to-cell cohesion in human vascular endothelial cells. In particular embodiments of the invention, the increase in cohesion is achieved by increasing the number of cell surface molecules involved in cell-cell cohesion. In other particular embodiments, the increase in cohesion is achieved by increasing the number of functional molecules (e.g., dephosphorylated β catenin) bridging cadherins to a cytoskeleton, or otherwise reducing the amount of dissociation of cadherin from the cytoskeleton of said human vascular endothelial cells.

In a particular aspect, the invention is a method of increasing cell-to-cell cohesion, said method comprising increasing the amount of cadherin of native vascular endothelial cells.

Cadherins are glycosylated polypeptides. Virtually all multicellular organisms including, for example, those found in mammalian, avian, amphibian and teleost cells have cadherins. For purposes of implementing the present this invention, the glycosylation will take place in a human endothelial cell, but the polypeptide backbone can be that coded for by any cadherin gene, as any such gene can be transfected into human endothelial cells. It is preferred, however, that the cadherins used to effectuate the present inventions be eukaryotic cadherins, preferably mammalian cadherins, more preferably human cadherins, most preferably N-cadherins, E-cadherins, P-cadherins, and VE-cadherins. There are numerous known human cadherins.

Cadherins are recognizable because:

1) they are transmembrane proteins located at cell-to-cell junctions and are Ca2+-dependent for purposes of establishing strong, stable, intercellular bonds;

2) they react with antibodies made against various cadherin peptides, for example human VE, N-, E-, and P-cadherins.

3) they share homology to each other across species, but can differ in their extracellular domains, so as to confer specificity on individual cadherins.

One type of antibody useful in identifying cadherins is the pan-cadherin antibodies (raised against synthetic peptides containing conserved cadherin regions such as carboxy-terminal sequences). They are generally available, for example from Sigma, MO and were

used in Example 3 below. However, any type of antibody or antibody fragment raised by any known method may be used in this context.

In another aspect, the invention is a cell-coated solid surface comprising:

a) a solid surface; and

b) a population of altered human vascular endothelial cells, said cells adhering to said surface, where compared to endothelial cells of equivalent origin, said altered cells (on the average) contain more cadherin per cell.

c) alteration of native vascular endothelial cells adjacent to a vascular device to express cadherin on an endothelial cell..

Any known cadherin or fragment may be used in this invention, however preferred cadherins are VE cadherin, E cadherin, P cadherin, and N cadherin. Cadherin fragments of this invention are any part of a cadherin molecule which is effective to increase cell-cell cohesion in an assay, such as the assay of Example 2. Also part of this invention are synthetic cadherins or synthetic cadherin fragments, which can be produced and assayed by known methods.

Also contemplated for use in this invention are engineered or artificial molecules (also made by known methods) which increase cell-cell cohesivity (as measured by an assay such as that of Example 2) when inserted into a cell membrane. The artificial molecule is capable of interacting with a cell's cytoskeleton when inserted in the cell's membrane, and is capable of recognizing another surface molecule on another cell to increase cell-cell cohesivity. The surface molecule recognized by the artificial molecule may be the same artificial molecule, another artificial molecule, or a natural molecule already present on the other cell's surface. The artificial molecule is preferably a protein, proteoglycan, or peptide. In one embodiment, the artificial molecule has an intracellular, membrane, and extracellular domain. The extracellular domain is capable of tight association with another cell. The structure of such domains is well known and the artificial molecule may be designed based on such structures. Similarly it is known to design binding regions. The artificial molecule may be inserted in the cell membrane by any known means, one means being to use known techniques of genetic engineering to obtain expression by the cell of the gene encoding the artificial molecule.

In another aspect, the invention is a population of altered native vascular endothelial cells adjacent to a vascular device in a human vascular system where, compared to endothelial cells of equivalent origin, said altered cells (on the average) contain more cadherin per cell, and a population of human vascular endothelial cells adjacent to a vascular device where, compared to endothelial cells of equivalent origin, said cells have an increased number of cadherins associated with a cytoskeleton.

Two cells are of equivalent origin if they originate from the same type of tissue in the same type of organism. For example, two cells are of equivalent origin if they both originate from a human aorta, even if the aortas were in two different persons. A change in the average amount of cadherin cell per cell is a necessary and sufficient condition for a cell to be altered for purposes of the cell-coated surface inventions described herein.

Also part of this invention is a method of populating a solid surface with nonhuman endothelial cells, said cells not rejected in humans, said process comprising increasing the cell-to-cell cohesion of said endothelial cells.

Also part of this invention is a method of populating a solid surface with nonhuman endothelial cells, especially vascular endothelial cells, said cells not rejected in humans, said process comprising increasing the amount of cadherin per cell. One means for populating a solid surface with nonhuman endothelial cells is to reduce the amount of dissociation of cadherin from the cytoskeleton of said nonhuman endothelial cells. As described above, this can be accomplished by reducing or eliminating the phosphorylation of a molecule associated with the adherens junction between the nonhuman endothelial cells, in particular β catenin. Phosphorylation may reduced or eliminated by use of any agent known to do so, i.e. any agent which is known to modify phosphorylation, in an amount effective to reduce or eliminate phosphorylation. Examples of such agents include corticosteroids, and enzyme inhibitors such as serine-threonine kinase inhibitors, protein tyrosine kinase inhibitors, and phosphatase inhibitors. Specifically, such agents include 6-dimethylaminopurine, stuarosporine, erbstatin, herbimycin A, genestein, tyrophostins, and vanadate (see for example Aono, et al. J. Cell. Biol. 1999 145:551-562 and Monier-Gavelle, et al. J. Cell Biol. 197 137:1663-1681).

In a related aspect, the invention is a cell-coated solid surface comprising:

a) a solid surface; and b) a population of altered human vascular endothelial cells, said cells adhering to said surface, where compared to endothelial cells of equivalent origin, said altered cells (on the average) contain more cadherin genes per cell, so as to cause increased expression of functional cadherin.

A solid surface of particular interest is the inner surface of a tubular graft, said inner surface defining (enclosing) an open-ended cavity that extends the length of the graft.

Another solid surface of particular interest is one within a catheter, valve, pump, or other device within or in contact with the vasculature (i.e., the arterial and/or venous system).

Other solid surfaces of interest are those of channels and chambers inside a macrochannel prosthetic delivery patch designed to store cells or pharmaceuticals for delivery via pores to adjacent tissue (or graft surface). (See, for example, U.S. patent 5,782,789, which is incorporated herein by reference.). Yet other solid surfaces of interest are those in an *ex vivo* device which is used outside the body, such as a heart-lung machine. In general, solid surfaces include any relevant biomedical device.

Solid surfaces of interest include both porous and nonporous surfaces. They also include living tissue, such as that of the arterial or venous system.

The methods and cell-coated surfaces of the present invention are useful for achieving better utilization of vascular grafts. They are also generally useful for any system that employs endothelial cell-coated surface susceptible to hydrodynamic shear. Such general situations include shaking containers coated with endothelial cells, continuous harvest systems for harvesting the products of endothelial cells on solid surfaces, and laminar shear devices.

Also part of this invention is a method for determining whether an agent reduces or eliminates phosphorylation of a molecule associated with the adherens junction between the human vascular endothelial cells, comprising:

a) adding said agent to a preparation of human vascular endothelial cells in the presence of a solid surface;

b) allowing said human vascular endothelial cells to populate the solid surface; and

c) determining the extent to which the human vascular endothelial cells have populated the

solid surface in the presence of the agent and in the absence of the agent, and if the former is greater than the latter, thereby determining whether the agent reduces or eliminates phosphorylation of a molecule associated with the adherens junction. By this means useful agents for accomplishing the methods of this invention can be obtained.

Brief Description of the Drawings

Figure 1. Schematic, perspective view, in partial section, of a vascular graft with endothelial cells on its inner surface or in neighboring vascular tissue.

Figure 2. Comparison of shear-induced detachmentof AT.2and MAT-LyLu from matrigel.

Figure 3. Kinetics of shear-induced detachment from matrigel of MAT-LyLu cells.

Figure 4. FACS analysis of the L cells and genetically engineered LN2 cell line. The mean channel fluorescence (mcf) corresponding to the amount of N-cadherin expression of L cells was 0.749 which was not significantly different from background fluorescence. The mcf of the LN2 cells was 26.5.

Figure 5. Phase contract photomicrographs of the membranes were obtained of both cell types of membranes exposed to flow for 3 hr or no-flow controls. The bare area of the membranes represent area of cell detachment.

Figure 6. Resistance to shear-induced detachment as a function of intercellular cohesivity. The more cohesive LN2 cells have greater cells remaining post-flow.

Figure 7. VE-cadherin immunoprecipitation in human aortic vs. human umbilical vein endothelial cells. Immunoprecipitation of protein extracts from early passage human endothelial cells with an anti VE cadherin antibody followed by gel electrophoresis and immunoblotting for VE-cadherin results in a doublet. No significant difference in VE-cadherin is present.

Figure 8. VE-cadherin and α, β, and γcatenin coprecipitation experiments. Immunoprecipitation of protein extracts from early passage human endothelial cells with an anti VE-cadherin antibody was followed by gel electrophoresis and immunoblotting for an α (102 kD), β (94 kD), and γ (85 kD) catenin. Only γ catenin is consistently coprecipitated.

Figure 9. VE-cadherin and α catenin coprecipitation vs. supernatant experiment. Immunoprecipitation of protein extracts from early passage human endothelial cells with an anti VE-cadherin antibody was followed by gel electrophoresis of the immunoprecipitated protein and the non_IP supernatant followed by immunoblotting for α catenin. This revealed that α catenin was present but not coprecipitated with VE-cadherin.

Figure 10. All cadherins and α, β, and γcatenin coprecipitation experiments. Immunoprecipitation of protein extracts from human aortic endothelial cells with an anti pan-cadherin antibody was followed by gel electrophoresis of the immunoprecipitated protein and the non_IP supernatant followed by immunoblotting for α catenin. This revealed that α catenin was co-precipitated with cadherin, and also present in the non-IP supernatant.

Detailed Description of the Invention

Abbreviations and glossary

EC's stands for endothelial cells.

Endothelial cells include cells derived from either stem cells or actual blood vessels, and both those naturally occurring in a human and those cells of endothelial origin growing outside a human.

Vascular endothelial cells are those of blood carrying vessels, e.g., aortic and other arterial cells, and veins.

VE-cadherin stands for vascular endothelial cadherin.

RT-PCR refers to the Reverse Transcription - Polymerase Chain Reaction method.

A cadherin gene is any DNA sequence in a cell where that sequence can be transcribed into RNA and subsequently translated into a cadherin polypeptide.

Reducing as in reducing phosphorylation means any decrease in the level of phosphorylation from its natural levels, to the point of elimination.

Biocompatible Graft Materials

Because the basis of the invention is improved cell-to-cell cohesion, rather than improved cell-to surface adhesion, the invention can utilize a broad range of biomaterials.

Such materials are non-toxic to cells, can adhere to host tissue, and are chemically stable in the environment, tubular graft or otherwise, that they are used. Preferred biomaterials will include, for example, those otherwise found to be preferred for vascular grafts. Presently preferred substrates in graft surgery are Gore-Tex® (which is e-PTFE or expanded-polytetrafluorethylene), Dacron® (polyethylene terepthalate), urethane, silicon, metals, degradable polymers, collegen, or bioengineered blood vessels. Specific metals are metals used in stents and other implantable devices such as artificial organs.

Cadherin amino acid and mRNA nucleotide sequences

The following information on human P-cadherin (placental cadherin) was obtained from GenBank, accession number NMN_001793, which in turn refers to Y. Shimoyama et al., J. Cell Biol. 109 (4 Pt 1), 1787-1794 (1989), and appears with the GenBank disclaimer that this is a provisional reference sequence record that has not been yet subject to human review and that the final curated reference sequence may be somewhat different from this one. The amino acid sequence following "translation=" is referred to herein as SEQ ID NO:1 and the nucleotide sequence as SEQ ID NO: 2.

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// (End of SEQ ID NO: 2)

Catenin amino acid and mRNA nucleotide sequences

Human catenin amino acid sequences and mRNA sequences (or corresponding cDNA seqeunces) are available in GenBank and the scientific literature. For example, D. L. Rimm et al., Biochemical and Biophysical Research Communications, vol. 203, 1691-1699 (1994), discloses cDNA and amino acid sequences for two forms of human α(E)-catenin.

Schematic illustration of the invention

Figure 1 schematically illustrates the invention. A tubular graft **1** is shown joined to two portions, **15** and **17**, of the same human aorta **3**. The Figure is highly schematic. For example, in practice, the graft is joined to the aorta by the use of stitching (not shown) or by endovascular attachment with stents, hooks, or glue. Also the length of the tubular graft, relative to its diameter or the length of aorta overlapping the graft, may be much greater than shown in the Figure.

In the Figure, part of the aorta **3** and part of the graft **1** has been removed to better view their internal surfaces. A first population **5** of altered endothelial cells is shown on the inner surface **7** of the graft **1**. Those cells originated from cells seeded on the surface **7**. A

second population **9** of altered endothelial cells is also shown on surface **7**. The second population **9** originated from endothelial cells that make up the inner surface **11** of the aorta **3**. Both populations **5** and **9** are shown as localized on a small portion of the surface **7**. Preferably the population of cells completely covers the surface **7**. A population **12** of altered native endothelial cells is shown as part of the inner surface of the aorta **3** adjacent to the graft device **1**. All the altered cells in the Figure show increased cell-to-cell cohesivity compared to unaltered cells.

Examples

The following examples are intended to illustrate the invention, not limit it.

Example 1

To understand why humans fail to endothelialize vascular grafts, we have tested the hypothesis that endothelial cell-cell cohesion is significantly reduced in human endothelial cells and that this gives rise to the inability of human EC's to resist shear-stress induced detachment from biomaterials. We have focused our attention on cadherin-mediated cell-cell cohesion on the premise that restoring normal cadherin function in human endothelial cells will restore the ability to endothelialize vascular grafts.

Our first test of this hypothesis was to demonstrate that human aortic endothelial cells are less cohesive than bovine aortic endothelial cells. To understand why human endothelial cells fail to migrate on and undergo shear induced detachment from biomaterials, we have utilized a new model for the evaluation of the physical properties of different cell types. This model measures the cohesive binding energies of aggregates of cells. There are two general factors that must be overcome for a cell to detach from a material. One is the energy of cell-material adhesion. The other is the energy of cell-cell cohesion. We have previously shown that changes in cell aggregate cohesivity, through alteration in cadherin expression, results in a change in the ability of cells to spread on a substrate in the absence of hemodynamic forces (Reference: Foty et al. Cancer Research 58:3586-3589; Foty and Steinberg, Cancer Research 57:5033-5036; type of cell: HT-1080, LLC; (HT1080=Human Fibrosarcoma; LLC = Lewis Lung Carcinoma). Others have demonstrated as well that cells that are more cohesive spread more slowly than cells that are less cohesive. However, the cells that are more cohesive spread as a sheet of cells with increased cell-cell contact compared to the faster moving less cohesive cells (Breviario et al., Arterioscler Thromb Vasc, vol 15, pp 1229-39, 1995). This increase in cohesivity makes it more difficult for cell detachment (Applicants, data). In addition, others have shown that interfering with cohesivity leads to shear stress-induced detachment of endothelial cells from biomaterials (Schnittler et al., Am. J. Physiol., 1997, vol 273 (5Pt2):H2396-405).

The cohesivity of human and bovine aortic endothelial cells was measured by tissue surface tensiometry, (Foty, et al. Development 1996 122:1611-1620 and Foty, et al. Phys. Rev. Lett. 1994 72:2298-2301). Spherical aggregates of cells were formed by: $3x10^6$ cells were trypsinized from subconfluent tissue culture plates and washed 2x in complete medium, $3x10^6$ cells in 3 mls. were allowed to recover from trypsinization by shaking in a 37°water bath at 120 rpm for 4 hours. Cells were the centrifuged @ 950 x g for 4 min. at RT in a round bottom glass tube . Pellets were then incubated O/N at 37°/5% CO_2. Pellets were cut into 1 mm^2 fragments. Fragments were placed in shaker bath for 48 hrs. Briefly, 200-300 μm diameter spherical aggregates were compressed between parallel plates in degassed CO_2-independent medium at 37°C. The more strongly the cells in such aggregates cohere, the greater will be the force with which they resist separation. Such aggregates also resist deformation. Using a recording electrobalance, the force exerted by the originally spherical aggregates upon the parallel plates was monitored as it decreased to a constant value following initial compression. The aggregates profile was recorded by videomicroscopy. By measuring the force of resistance to the deforming force and the geometry of the compressed aggregate, cohesivity was calculated using the Young-Laplace equation (see for example Davies and Rideal, Interface Phenomena. New York, Academic Press 1983).

The absence of cohesivity of the human aortic endothelial cells was such that aggregates cohered too weakly to allow measurement of cohesivity. However, the cohesivity of the smooth muscle cells from both species tested was measurable as noted in **Table 1**. The results show that these cohesivities are high and within the range of tissues, such as the embryonic chick limb bud, considered as very adhesive.

Example 2

The second methodology we employed was a functional aggregation assay. To assess function of cadherins junctions, an aggregation assay was performed comparing the formation of cellular aggregates over time among bovine aortic (BAEC), human aortic (HAEC) and umbilical vein (HUVEC) endothelial cells. All three cell types were maintained in tissue culture, then detached by trypsinization and placed in a shaking culture vessel, shaken at an

initial concentration of cells of **1** x10^6 cells per ml, in a solution volume of 3 ml, in a glass container in MCDB 131/ 2%FCS/ ENDOquot Bullit kit (Clonetics). After 3 hours in shaking cultures, both BAECs and HUVECs formed cellular aggregates while HAECs demonstrated minimal aggregation, again reflecting decreased cohesivity.

The results of this Example and Example 1 demonstrate that there is a clear difference in cohesivity between human aortic endothelial cells and bovine aortic endothelial cells. In contrast, human umbilical vein endothelial cells tend to behave more like cells of non-human origin.

Example 3

The next hypothesis that we have evaluated was that the difference in cohesivity among endothelial cells of different origins could be explained by differences in cadherins present on the cell surface. Specifically, that human aortic endothelial cells would have decreased surface cadherins. All three cell types were maintained in culture. Cells were removed from the flasks by scraping and lysed for Western blotting. Experiments using a pan-cadherin antibody revealed that both BAEC and HUVEC expressed similar levels of cadherin, but HAEC expressed substantially less (50% reduction vs. BAEC, 53% vs. HUVES, ($p<.05$ by ANOVA).

This study confirmed that the human aortic endothelial cells were deficient in cadherins relative to the more cohesive bovine and umbilical vein cells.

Example 4

To further assess the cause for decreased cohesivity, we have studied other components of the adherens junctions. Catenins are proteins that are linked to the transmembrane subunits of cadherins and maximize binding energy by allowing interaction between cadherins and the actin cytoskeleton. To identify the etiology of differences in cohesivity between human and bovine aortic ECs, α, β, and γ catenin expression was determined by western blotting. No differences in immunoreactivity among catenins were noted between human aortic endothelial cells and the endothelial cells from other species.

Of interest are the causes of cadherin down-regulation in human aortic endothelial cells. We have confirmed that the mRNA for VE-Cadherin is present in human aortic endothelial cells by RT-PCR. Also of interest is whether cadherin down-regulation is under transcriptional or translational control.

In one approach, one clones the gene for VE-cadherin from human endothelial cells and uses that gene to restore VE-cadherin expression in human endothelial cells with a view to increasing cohesivity and improving endothelialization of vascular grafts under shear flow conditions.

Example 5

One increases the cohesivity of human aortic endothelial cells by transfection of various cDNAs encoding several cadherins, including E-, N, P-, or VE-cadherin. One generates cell lines in which cadherin expression is under the control of an inducible promoter. The Ecdysone-Inducible Mammalian Expression System (Invitrogen) is based on the molting induction system found in Drosophila, but has been modified for inducible expression in mammalian cells. Maximal expression levels in this system are quite high, and have been reported to induce protein expression over 200 fold above basal level. In the Ecdysone-Inducible Mammalian Expression System, both subunits of a functional ecdysone receptor from Drosophila are constitutively expressed from the regulator vector pVgRXR. The ecdysone-responsive promoter (pΔHSP) which will ultimately drive the expression of the cadherin gene--is located on a second inducible expression vector. Mammalian cells are co-transfected with the inducible expression vector containing the cadherin gene along with pVgRXR by electroporation utilizing a commercially available gene transfer apparatus (Bio-Rad Gene Pulser II System). Electroporation continues to be one of the most efficient means of introducing genes into mammalian cells. Electroporation is a physical process that transiently permeabilizes eukaryotic cell membranes with an electrical pulse, thus permitting cell uptake of a wide variety of biological molecules. One optimizes the electroporation pulse for human endothelial cells by conducting experiments in which cDNA encoding green fluorescent protein (GFP) is electroporated into cells under various conditions of applied

voltage and capacitance, various cDNA concentrations as well as sample volume. Once parameters are optimized, one employ similar conditions for electroporation of cadherin cDNA. Transfected cells are selected in medium containing 500 μg/ml Zeocin and 800 μg/ml G418. Stabily transfected cells are placed in medium containing Ponasterone A and FACS sorted for high levels of cadherin expression.

Alternatively, one employs more conventional transfection methods allowing for efficient introduction of cadherin genes into endothelial cells. These may include calcium phosphate precipitation, lipofection, or adenovirus/retrovirus mediated infection. A somewhat less conventional approach includes use of a biolistic Gene Gun particle delivery system (BioRad). The Helios Gene Gun is a convenient, hand-held device that provides rapid and direct gene transfer into a range of targets under in vivo conditions. The unit employs an adjustable, low-pressure helium pulse to sweep DNA- or RNA-coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target. Cadherin cDNAs can also be introduced in vivo by admixing with slow release polymers incorporated into the vascular graft biomaterial at the site of anastamosis.

Example 6

As we have demonstrated in previous publication, one can measure cell-cell cohesivity by tissue surface tensiometry. We propose that one can measure cell-substratum adhesion by shear flow induced detachment. As proof of principle, we conducted several preliminary experiments to assess the efficacy of this technique in measuring cell-substratum adhesion of two prostate cancer cell lines, In the first experiment, we compared the shear-induced detachment of the AT.2 and MAT-LyLu cell lines as a function of shear stress. In this experiment, cells were allowed to attach for 24 hours prior to the application of shear stress. We found that the less invasive, and more cohesive AT.2 cell line was better able to resist shear-induced detachment from matrigel than did the highly invasive and less cohesive MAT-LyLu (**Figure 2** shows the extent to which the surface is coated with cells after exposure to various shear forces). The next experiment asked whether we could address questions on the kinetics of cell attachment to substrate. Here we allowed MAT-LyLu cells to

attach for either 1, 4 or 18 hours prior to application of shear stress then counted the number of cells still attached to substrate after 4 hours of 30 dyne/cm flow. The results showed a time-of-attachment-dependent relationship described best by a 2nd order polynomial (**Figure 3**).

Table 1

Cell Type	Mean Cohesivity (dyne/cm±SE)
Human Aortic Endothelial	<1
Bovine Aortic Endothelial	15.3±3.6
Human Aortic Smooth Muscle	20.9±0.4
Bovine Aortic Smooth Muscle	11.8±2.9
Chick Limb Bud	20.1±0.5

Example 7

To investigate the possible mechanisms responsible for shear induced endothelial cell detachment from biomaterials, one must consider the forces that resist this process: cell-material adhesion and cell-cell cohesion (the strength of cell-cell attachment). We have hypothesized that improved cell-cell cohesion could be an additional force that must be overcome for shear induced cell detachment to occur. We have demonstrated that differences in cell-cell cohesivity exist among endothelial cells obtained from human sort a versus bovine aorta (an example of a species that is able to endothelialize prosthetic grafts (Foty, et al. Mol. Biol. Cell. 2000:11 abstract). Human endothelial cells were noted to have significantly impaired cohesivity as compared to bovine endothelial cells. This cohesive energy is determined by the function of the adherens junctions between cells (Nagafuchi, Current Opin. Cell Biol. 2001, 13:600-603). The adherens junction is created by the presence of transmembrane cadherin molecules linked to the actin cytoskeleton. The cadherins from adjacent cells may link to form high-energy bonds.

It has been suggested that general disturbance of cell-cell interactions could induce shear related loss of the intercellular bonds of cells (Schnittler, et al. Am. J. Physiol. 1997 273:H2396-

2405). However, the specific role of cadherin mediated cell-cell cohesivity in preventing shear-induced detachment of cells from materials has not been elucidated. In this study, we tested the hypothesis that cadherin mediated cell-cell cohesivity is an independent determinant of the ability of cells to resist shear-induced detachment.

To test our hypothesis, we utilized cells that differed only in cadherin mediated cell-cell cohesivity. A mouse fibroblast cell line (L 929:ATCC) was chosen since they are a non-cohesive cell line that do not express cadherin. We genetically engineered cells to express N-cadherin. In 300μl of serum-free RPMI 1640, 10mM dextrose/0.1mM dithiothreitol, $4x10^6$ L cells were transferred to a 0.4cm electroporation cuvette. Cells were transfected with 40 μg of pMiwcN chicken N-cadherin expression vector (Fujimori, et al. Development 1990 110:97-104) along with 4 μg of pZeoSV (Invitrogen, Carlsbad, CA) for Zeocin selection using a Bio-Rad Gene Pulser II gene transfer apparatus at 0.350 kV and 500μF. Transfected cells were diluted 1/100 and plated into medium containing 300μg/ml Zeocin. Resistant cells were grown to confluence, detached by trypsin/Ca2+ and stained by incubation in 10μg/ml anti-chicken-N-Cadherin antibody (NCD2, Zymed, San Francisco, CA) on ice for 45 minutes. After several washes in Hanks' balanced salt solution, cells were mixed with a fluorescein isothiocyanate-conjugated secondary antibody and placed on ice for 30 min. N-cadherin-expressing cells were autocloned into 96 well plates using the Clone-Cyt Integrated Deposition System (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Positive clones were re-analyzed by flow cytometry. Two cell lines expressing different levels of N-cadherin (Lncad-2, and Lncad-4) were propagated. These genetically engineered cells produce different levels of N-cadherin and have a linear relationship between cohesivity and cadherin expression. The LN2 cell line was used for all subsequent studies. Aggregates of LN2 cells were noted to have a measurable energy of cohesivity by tissue surface tensiometry of 1.9 dynes/cm(Ryan, et al PNAS 2001 98:4323-4327). Untransfected L cells do not form cellular aggregates and therefore precluded the use of tissue surface tensiometry.

To study shear induced detachment, we developed an assay in which approximately 50% of the non-cohesive L cells would detach during flow. Initial studies demonstrated that L-cells, despite being the least cohesive, adhered strongly to the biomaterial and resisted detachment under 30 Dynes/cm^2 for 24 hr. Since L cells are naturally extremely adhesive to surfaces, we

needed to "passivate" the membranes prior to cell seeding to decrease integrin mediated cell adhesion. Folkman et al. had shown that a thin coating of poly HEMA at different dilutions reduces the ability of the bovine aortic endothelial cells to adhere to tissue culture plastic (Folkman and Moscona, Nature 1978 273:345-349). We dissolved 1.2 g of poly HEMA in 10 ml of 95% ethanol over night at 37 degrees. This stock solution was dissolved in 95% ethanol to obtain 2 x 10^{-1},10^{-1},2 x 10^{-2},10^{-2},2 x 10^{-3} dilutions. The prosthetic material used as substrate was Dacron ® (polyethylene terepthalate). Commercially available Dacron membranes with a pore size of 0.45 microns were used (Cyclopore membrane, Falcon cell culture insert: Becton Dickenson, Franklin Lakes, NJ). We identified that a dilution of 1:100 resulted in 50% detachment

The L and LN2 cells were plated at confluent densities (1x 10^6 cells/membrane) on the poly HEMA coated Dacron membranes. After 3 hr. the membranes were placed into a parallel plate apparatus and exposed to flow resulting in 30 dynes/cm^2 of shear stress for 3 hr. This apparatus has been previously described (Nackman, et al. Surgery 1998 124:353-360). Other membranes were not exposed to shear stress to serve as no flow controls (N=3-5 membranes per call type per shear stress). Post flow, images of the remaining cells on the membranes were obtained by phase contrast microscopy. The adherent cells were released from the membrane with trypsin and manually counted with a hemocytometer. The percentage of cells remaining was determined by normalizing cells counts to the no flow controls for each cell type. ANOVA was used to determine if a statistically significant difference in resistance to shear-induced detachment among the L and LN2 cell types was present.

Analysis of L and LN2 cells by flow cytometry revealed that the LN2 cells expressed substantial amounts of N-cadherin on the cell membrane. Figure 1 demonstrates the mean channel fluorescence of the two cell populations. The left peak represents the basal fluorescence of the L cells. The peak on the right side of the graph reveals the presence of N-cadherin on the LN2 cells.

After 3 hr. of 30 dynes/cm^2 of shear stress, significant numbers of L cells did detach from the membrane as compared with the more cohesive LN2 cells and the no flow controls. The post flow images revealed significantly larger area of gaps in L cell coverage of the membranes as compared with the LN2 cells (Figure 2). Cell counts revealed that 87.4$\pm$4.4% (mean$\pm$sem) of

LN2 cells remained on the membrane as compared with the no flow controls. The less cohesive L cells had significantly fewer cells present, 45.4±10.0%, P<.05 (Figure 3).

Adherens junctions are responsible for cell-cell cohesion and are common to many cells including endothelial cells (Navarro, et al. J. Bio. Chem. 1995 270:30965-30972). These junctions connect cells and provide them with mechanical stability. Several key proteins are know to mediate the function of adherens junctions, including the Ca^{+2} dependent cadherin family, catenins, and other proteins that link to the actin cytoskeleton. This study demonstrates specifically that cadherin mediated cell-cell cohesivity has an essential role in the ability of cells to resist shearinduced detachment. The less cohesive L cells only differ from the more cohesive LN2 cells by N-cadherin expression. This proof of principle that cell-cell cohesivity affects the ability of a population of cells to resist shear induced detachment may be an important finding related to the failure of human endothelial cells to resist shear-induced detachment and to heal vascular grafts.

This study differs from other studies in the manner of evaluating the specific role of cadherin mediated cell-cell cohesion in preventing shear induced detachment. Schnittler et al. exposed human umbilical vein endothelial cells cultured on gelatin coated glass to shear stress after treatment with a high concentration of EGTA (3nM)(Schnittler, et al.). The treatment with EGTA resulted in depletion of calcium. The investigators noted that over time calcium depletion resulted in the loss of VE-cadherin, ß and Y catenin by immunostaining. This was associated with formation of gaps between cells induced by shear stress. However, cell detachment was not identified or quantified. Removal of calcium may have also alterned integrin function and cell shape (Nebe, et al. Exp. CEll. Res. 1996 229:100-110). Loss of integrin function could also have affected the ability of cells to respond to shear stress (Girard and Nerem, Front. Med. Biol. Eng. 19934 5:31-36). Furthermore, integrins may also have a significant role in cell-cell attachments (Lampugnani, et al. J. Cell Biol. 1991 112:479-490 and Kaufman, et al. J. Cell Biol. 1989 109:1807-1815). Our study isolates the role of cadherin mediated cohesivity from the integrin mediated adhesivity in shear induced detachment.

To study the role of cadherin function as an independent factor in maintaining cell-cell cohesivity under shear stress, mouse fibroblast cells that express different levels of cadherin were used. In this experiment, we used an immortalized, originally non-cohesive mouse fibroblast cell

line and an N-cadherin transfectant to test our proof of principle. The ideal cell type for this experiment would be to use endothelial cells obtained from human tissue. Unfortunately, no immortalized cell line of human arterial endothelial cells currently exists. We are currently in the process of generating such a line. Future studies will focus on increasing human endothelial cell cohesivity, and determining if this results in a sufficient alteration of human endothelial cell behavior to adapt to shear stress in a manner similar to endothelial cells of other species.

Example 8

To elucidate the mechanism responsible for failure of human endothelial cell cohesion, we continued our evaluation of differences in cadherin expression among bovine and human aortic and umbilical vein endothelial cells. We tested the hypothesis that human aortic endothelial cells would have decreased VE-cadherin present on the cell surface as compared to human umbilical vein endothelial cells. Immunoprecipitation experiments demonstrated that there were not significant differences in VE-cadherin despite the previously described 2x increase in total cadherin reactive with a pancadherin antibody (Figure 7). This implicates that other non VE-cadherins may be significantly decreased on human aortic endothelial cells as compared with both bovine and human umbilical vein endothelial cells. We performed direct comparisons of VE-cadherin presence on porcine and human aortic endothelial cells and again found no differences.

β catenin phosphorylation is a mechanism through which the adherens junction may be rendered non-functional. After phosphorylation the α catenin complex dissociates from the cadherin. We have also tested the hypothesis that the failure of human endothelial cell cohesivity may be due to lack of function of the adherens junction. Previously we defined no difference in immunoreactive α, β, and γ catenins between human aortic, human umbilical, and bovine aortic endothelial cells. We have now demonstrated that α catenin has become disassociated from the adherens complex in human aortic and umbilical vein endothelial cells. Immunoprecipitation experiments were performed with anti VE-cadherin antibody followed by immunoblotting for α, β, and γ catenins with anti-catenin antibodies. The β and γ catenins were brought down with the VE-cadherin, however, α catenin was not (Figure 8).

The α catenin remained in the supernatant of the immunoprecipitation experiment for VE-cadherin (Figure 9). These data support the hypothesis that the VE cadherin containing adherens junctions of human endothelial cells lack function. Whens the immunoprecipitation experiments were repeated using an anti pan-cadherin antibody rather than the anti VE cadherin antibody, α cadherin was brought down while some remained in the supernatant (Figure 10). This implies the possible differential regulation of the adherens junctions based on type of related cadherin. Immunoprecipitation studies for β catenin followed by immunoblotting for the presence of phosphorylation reveals significantly increased phosphorylation of the β catenin of human aortic endothelial cells as compared with porcine aortic endothelial cells and human aortic smooth muscle cells.

This reveals a mechanism responsible for decreased function of the adherens junction in human endothelial cells.